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שהופקדו לכתחילה
עם הבקשה לפטנט
לפי הפרטים הרשומים
בעמוד הראשון של
הנספח.

This 24 -06- 1998 היום

מ. בשרי
ממונה על הבוחנים

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חוק הפטנטים, תשכ"ז - 1967
PATENTS LAW, 5727-1967

בקשה לפטנט
Application For Patent

לשימוש הלשכה -
For Office Use

מספר: Number	121349
תאריך: Date	21-07-1997
הוקדם/נדחה Ante/Post-dated	

אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
I, (Name and address of applicant, and in case of body corporate-place of incorporation)

ידע חברה למחקר ופתוח בע"מ, ליד מכון ויצמן למדע ת"ד 95, רחובות 76100, ישראל
YEDA RESEARCH AND DEVELOPMENT COMPANY, at the Weizman Institute of Science, P.O.Box 95, Rehovot 76100, Israel

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(באנגלית) (English)
Delivery of substances to injured site of the central nervous system

מבקש בזאת כי ינתן לי עליה פטנט hereby apply for a patent to be granted to me in respect thereof.

* בקשת חלוקה — Application of Division	* בקשת פטנט מוסף — Appl. for Patent of Addition	* דרישת דין קדימה Priority Claim		
מבקשת פטנט from application	* לבקשה/לפטנט to Parent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האיגוד Convention Country
No. מס'	No. מס'			
dated מיום	dated מיום			

* יפוי כח : מיוחד P.O.A. : Special

עוד יוגש הוגש בענין קודם filed on a previous case

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Signature of Applicant

For the Applicants,
DR. REINHOLD COHN AND PARTNERS
By : -

היום 20th בחודש July שנת 1997 This of the year

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סופס זה כשהוא מובטע בחותם לשכת הפטנטים ומושלם במספר ובתאריך ההגשה, הנו אישור להגשת הבקשה שפרטיה רשומים לעיל.
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הושטה של חומרים לאתר פגוע של מערכת העצבים המרכזית

Delivery of substances to injured site of the central nervous system

Yeda Research and
Development Company Ltd.

C. 99973

DELIVERY OF SUBSTANCES TO INJURED SITE OF THE CENTRAL NERVOUS SYSTEM

FIELD OF THE INVENTION

The present invention is generally in the field of therapy and diagnosis and is concerned with the treatment and diagnosis of damages in the central nervous system (CNS). The term *"CNS damages"* will be used herein to denote a variety of CNS injuries and genetic disorders which adversely affect the physiology of the CNS or impair neural pathways in the CNS. CNS damages include genetic degenerative diseases, e.g. Alzheimer's disease and Parkinson's disease; ischemias, i.e. loss of certain cells within the CNS as a result of impairment in blood flow, e.g. as a result of a stroke; various physical injuries, e.g. result of a strong impact on the skull such as a car accident, damages resulting from penetration of objects into the skull such as a bullet; inflammations within the CNS; etc.

Provided by the present invention are therapeutic methods for the treatment of CNS damages as well as preparations for use in such methods. Also provided by the present invention are diagnostic methods and kit reagents for use in such methods.

PRIOR ART

The following is a list of prior art considered to be pertinent as a background to the present disclosure:

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15. Wekerle, H., Experimental autoimmune encephalomyelitis as a model of immune mediated CNS disease, *Curr. Opin. Neurobiol.*, 3:779–784 (1993).

16. Ben–Nun, A. & Cohen, I.R., Experimental Auto Immune Encephalo–myelitis (EAE) mediated by T cell lines: process of selection of lines and characterization of the cells, *Journal of Immunology*, 129:303–308 (1982).

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These references will be referred to in the text by indicating their number in the above list within brackets.

BACKGROUND OF THE INVENTION

Traumatic injuries in the CNS are known to lead to an invasion of blood derived macrophages as well as activation of microglia within the brain [1–9]. The invasion is however delayed and more limited in its scope as compared to that in peripheral nerves traumas [2,10,13]. In addition, the duration of events associated with the acute phase of the injury is prolonged in the CNS as compared to the peripheral nerve system (PNS) and several weeks after injury, numerous activated macrophages and microglia are found in the CNS, while only a few are detectable in PNS nerves at such time after injury [12,14].

It is known that activated T–cells are able to cross the blood–brain barrier (BBB) and recognize epitopes in the CNS (Wekerle [15], but the role of T–cells in enhancing or impeding CNS nerve regeneration, is not yet known.

GENERAL DESCRIPTION OF THE INVENTION

The present invention is based on the very surprising finding that activated T–cells, regardless of the epitope recognized by them, accumulate at a site of CNS damage. The present invention is further based on the realization that activated T–cells with a non–self epitope specificity may thus be used to advantage in delivering various substances to a damaged site in the CNS for various therapeutic and diagnostic applications.

Provided in accordance with the invention is a method for the delivery of a substance to a site of damage in an individual's central nervous system (CNS), comprising activating T–cells which either carry or can produce said substance, and allowing said cells to migrate within the individual's body to reach said site.

The present invention also provides T–cells carrying or capable of producing a substance in need at a site of CNS damage, said substance being a substance which is not naturally carried or produced by the T–cells.

The T–cells may be kept frozen under conditions so that after thawing, the cells become viable. The T–cells may also be formulated in a variety of compositions, e.g. in a medium which maintains viability of the T–cells for a time until they are inoculated into the body and which medium is also pharmaceutically acceptable for parenteral administration.

The T–cells may be activated by exposure of the cells to a variety of natural and synthetic antigens and epitopes, including super antigens such as LPS (lipopolysaccharide) which activate a large number of T–cell clones, or antigens which activate the number of T–cell clones, such as antigens used in the experiments described herein, and many others. During the *ex vivo* activation phase of the T–cells, a variety of growth promoting

substances may be added to the T-cell containing medium including interleukins such as IL-2.

The T-cells are preferably syngeneic T-cells, particularly T-cells withdrawn from the same individual but they may also be T-cells obtained from HLA identical individuals. In addition, the T-cells may also at times be allogeneic T-cells, e.g. a pooled T-cell preparation obtained from a blood bank. The use of allogeneic T-cells is applicable particularly for a variety of one-time treatments, e.g., delivery of T-cells to a site of CNS damage for diagnostic purposes, for an acute one time therapy, etc.

The T-cells may be used for the delivery of a variety of substances to a damaged site of the CNS, including substances with a diagnostic utility, as well as substances having a therapeutic utility.

Also provided by the present invention is a method for diagnosis of damage in the CNS of an individual, comprising inoculating the individual with activated T-cells carrying a detectable marker substance, allowing time for migration of the T-cells to a site of damage in the CNS and then detecting the presence and location of the marker substance in the individual's CNS. Detection of the presence and location of such T-cells may be by a variety of imaging techniques depending on the marker substance. For example, if the T-cells carry metal particles, this may be achieved by magnetic resonance imaging (MRI) techniques; where the substance is a radioactive substance, the place of location may be achieved by a variety of imaging devices based on the detection of radioactivity.

For diagnostic application, T-cells will typically be loaded with contrasting agents, e.g., with metal such as gold particles, with radioactive markers, with gadolinium complexes (contrast agents for MRI), etc. The loading of the T-cells with such substance will be *ex vivo*, and after loading, the T-cells will be activated and then inoculated into the blood system, whereby at least a portion will localize at a site of damage in the CNS.

Loading of the T-cells with such substance may be achieved by a variety of means known *per se*. For example, T-cells may be incubated

in a suspension comprising the metal particles and the loading will be a result of the spontaneous internalization of such particles into the cell's cytosol. Such substances may also be introduced into the cells by a variety of electrophoretic techniques. Loading of T–cells with a radioactive marker may be achieved by incubating cells with a radioactive metabolic precursor, e.g., for example, labeled with a radioactive marker having a short half life such as ^{131}Iodine and ^{99}Technecium, etc.

The invention thus further provides a method for the treatment of damage in an individual's CNS comprising inoculating the individual with activated T–cells carrying or producing a substance in need at a site of CNS damage to ameliorate the damage or symptoms associated therewith.

Substances with therapeutic utility include a variety of growth factors which promote nerve regeneration, substances lacking at the site of damage, e.g. a neurotransmitter, an anti–inflammatory substance, etc. For therapeutic application, the T–cells may be treated *in vitro* to insert therein a DNA sequence which either encodes said substance, encodes an enzyme which catalyzes said substance, or encodes a regulatory product the production of which brings to expression of said substance in the T–cells, the DNA sequence being under expression control such that it is either expressed continuously or induced to expression as a result of exposure of the T–cells to a microenvironment of a kind present at the site of DNA damage, typically to yield secretion of expression product of said DNA sequence. Examples are DNA sequences encoding the nerve growth factor (NGF), DNA sequences encoding enzymes which play a role in CNS nerve regeneration such as the enzyme transglutaminase, DNA sequences encoding an enzyme which catalyzes the production of a neurotransmitter, e.g. enzymes involved in the catalysis of acetylcholine or dopamine, etc. As a result, the T–cells which will localize at the site of damage in the CNS will produce and secrete the needed substances at the site.

The *"genetically engineered"* cells, i.e. the cells inserted with said DNA sequences, may be selected by a number of means, e.g. detecting production of said substance, use of a variety of expression markers, etc.

The T-cells may both be stably transfected with said DNA sequences or may be transiently transfected. Transient transfection may at times suffice, particularly for acute one-time therapeutic regimes.

Therapeutic utilities of the invention include the induction of regeneration of damaged CNS nerves; delivery of missing neurotransmitters to damaged site at a variety of degenerative CNS disorders, such as delivery of acetylcholine into a degenerated site of the CNS in patients suffering from Alzheimer's disease, delivery of dopamine to degenerative sites in Parkinson's disease patients; etc.

The T-cells of the invention, whether used for therapeutic or diagnostics, may be administered to the individual by intravenous or intraperitoneal injection.

The present invention will now be illustrated further by a description of experiments conducted in accordance with the invention, with occasional reference to the annexed drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a montage of epifluorescence micrographs of serial sections of an optic nerve excised fourteen days after a controlled crush injury. At the time of crush, the tested animals were injected intra-peritoneally [ip] with $5x10^5$ T-cells primed to the antigen myelin basic protein (MBP) (T_{MBP}) that were labeled with Hoechst stain. One section was stained with anti GFAP (antibodies against glial fibrillary acidic protein) to visualize the injury site (lack of GFAP positive astrocytes) and photographed using filters that detect rhodamine and the others were photographed using filters that detect Hoechst stain to visualize the labeled cells (these appear as white dots). The figure shows that injected T_{MBP} cells selectively accumulate at the injury site (I) and just distal (D) to the injury.

Fig. 2 shows a higher magnification of the site of optic nerve injury shown in Fig. 1, showing the large concentration of injected cells localized in the site of injury.

Fig. 3 shows a serial section through non-injured optic nerve.

Fig. 4 is a graphical representation of the number of T–cells, of two different T–cell clones primed with antigens MBP and ovalbumin (OVA) (T_{MBP} and T_{OVA}) after injury, at various time intervals following injury. T_{MBP} and T_{OVA} cells were injected into animals at the time of optic nerve crush, ipsolateral and contralateral nerves were then removed and prepared for microscopy at days 3, 7, 14 and 21. The figure shows that T–cells accumulated at the site of injury, independent on the antigen they were primed with (each result is an average of 5 different experiments; the bar shows the standard deviation).

Fig. 5 shows accumulation of T–cells measured immunochemically using antibodies to T–cell receptors. The results show a comparison of the number of accumulated cells in injured optic nerve (ON) and in non–injured Optic nerve ("T_{MBP}" and "T_{OVA}" – as above).

Fig. 6 shows accumulation of T–cells in injured and non–injured optic nerve after various treatment protocols. T–cells specific to MBP (T_{MBP}) were injected either immediately after nerve injury (T_{MBP} Cell Injection = 0) or 14 days after injury (T_{MBP} Cell Injection = 14) and their accumulation at the optic nerve was analyzed either 7 days (nerve excision – day 7) or 21 days (nerve excision = day 21) after injury.

DESCRIPTION OF EXPERIMENTS

1. METHODS

Animals

Inbred Lewis rats were supplied by Harlan Olac (Bicester, UK), matched for age (8–12) and sex and housed four to a cage in a light and temperature controlled room.

Proteins used for T–cell stimulation

MBP Ag was prepared from the spinal cords of guinea pig and OVA Ag was prepared from chicken ova as previously described [16].

Activation and propagation of T–cell clones

T–cell clones, generated by peripheral lymph node cells and collected 10 days after either MBP (myelin basic protein) or OVA (ovalbumin) injection into animals, were activated by restimulation *in vitro* with either MBP or OVA antigen (10 μg/ml) on a feeder layer of irradiated (2000 rds) thymus cells (107/ml) in Petri dishes (10 ml/dish) in proliferation medium. Proliferation medium contained Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Israel) supplemented with 2 mM L–glutamine (Sigma, USA), $5x10^{-5}$ M 2–mercaptoethanol (Sigma), penicillin (100 IU/ml; Biological industries), streptomycin (100 μg/ml; Biological Industries), sodium pyruvate (1 mV; Biological Industries), non–essential amino acids (1 ml/100 ml; Biological Industries) and autologous rat serum 1% (vol/vol). After incubation for 48–72 hrs. at 37°C, 90% relative humidity and 7.5% CO_2, the cells were transferred into propagation medium consisting of 10% fetal calf serum (FCS) (vol/vol) and 10% T–cell growth factor (TCGF), which is from the supernatant of Con–A–stimulated spleen cells. Cells were grown in propagation medium for 4–10 days before restimulation.

Labeling of T–cells

T–cells were washed and suspended in 10.7 μM Hoechst 33342 stain (Molecular Probes, USA) for 10 minutes at 37°C. The cells were washed two times with 50 ml volumes of PBS and then resuspended at $5x10^6$ cells/ml on ice until being injected.

Crush injury of rap optic nerve

Crush injuries were performed as previously described [17]. Briefly, rats were deeply anesthetized by intraperitoneal (ip) injection of xylazine (10 mg/kg; Rompun) and ketamine (50 mg/kg; Vetalar). Under a binocular operating microscope, a lateral canthotomy was performed in the right eye and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed

intraorbitally by blunt dissection. The optic nerve was given a moderate crush injury 2 mm from the eye, using a calibrated cross–action forceps. The contralateral nerve was left undisturbed and was used as a control in the experiments.

Sectioning of nerves

At the specified time points, rats were sacrificed by over–anesthesia with ether and their optic nerves were surgically removed, immersed in Tissue–Tek (Miles Inc., USA) and frozen in liquid nitrogen cooled iso–pentane (BDH, UK). Then nerves were transferred to dry ice nd then stored at –70°C until sectioning. Longitudinal cryostat sections (20 μm thick) of the nerves were picked up onto gelatin–coated glass slides (4 per slide) and frozen at –20°C until viewed or prepared for fluorescence staining.

Immunolabeling of sections

Sections were thawed and fixed in ethanol for 10 minutes at room temperature, washed twice with double–distilled water (ddH_2O), and incubated for 3 minutes in PBS containing 0.05% polyoxyethylene–sorbitan monolaurate (Tween–20; Sigma, USA). The sections were then incubated overnight at 4°C with a monoclonal antibody (ED1; 1:400; Serotec, UK) directed against macrophages and antibodies against glial fibrillary acidic protein (GFAP; 1:100; BioMakor), all diluted in PBS containing 3% FCS. The sections were then washed two times with PBS containing 0.05% Tween–20 and incubated with mouse anti $F(ab')_2$ conjugated to either fluorescein isothiocyanate (FITC; BioMakor) or tetramethyl rhodamine isothiocyanate (TRITC; BioMakor) at a dilution of 1:50 for 1 hour. The sections were washed with PBS containing Tween–20 and treated with glycerol containing 1,4–diazobicyclo–(2,2,2) octane (Sigma), to inhibit quenching of fluorescence. The sections were viewed with a Zeiss Universal fluorescence microscope using filters that detect TRITC, FITC, and Hoechst stains. Detection of accumulated T–cells by antibodies disected to T–cell

receptors was done as above except that the incubation was carried out for 1 hour with the primary antibodies (anti-T-cell receptors).

Data analysis of T-cells in nerve sections

Nerves from various time points were prepared and sectioned. Hoechst labeled nuclei were counted in each section using the fluorescence microscope. Five sections were counted per time point and the numbers were averaged.

2. RESULTS

T-cell clones primed to MBP (T_{MBP}) were stimulated with MBP for 2 days before being labeled with Hoechst stain and injected into animals ip at the time of injury. At 3, 7, 14 and 21 days after injury, the nerves were excised, cryosectioned and analyzed microscopically for the presence of labeled T-cells. T_{MBP} cells were detected in the injured optic nerves at day 3 and accumulated until a peak at day 14 (Fig. 1). Large clusters of T_{MBP} cells were observed at the injury site and fewer individual cells were seen proximal and distal (Fig. 2). Four weeks after injury, labeled T-cells were still detectable in the degenerating optic nerves. No T-cells were found in the non-injured optic nerves (Fig. 3), non-injured sciatic nerve or injured sciatic nerve at any time after injury. Labeled T-cells were occasionally found in capillaries and in connective tissue but were not concentrated or localized into any specific areas. T-cells that were not prestimulated with antigen did not accumulate in any of the nerves, including damaged nerves.

The accumulation of T_{MBP} cells in injured CNS, but not in injured PNS, suggests that there might be some specific interaction between the primed T cells and the CNS tissue from which the MBP antigen was originally derived. To determine whether the injured CNS interacted with T-cells in general, or specifically with T-cells primed with a CNS antigen, the previous experiments were repeated using a clone that responds to chick ovalbumin (T_{OVA}). Rats were injected with a labeled T_{OVA} clone pre-

stimulated with ovalbumin (OVA) using the same protocol as with the T_{MBP} cells. The labeled T_{OVA} cells accumulated in injured optic nerve, and the pattern of accumulation was similar to that of the T_{MBP} cells. Labeled T_{OVA} and T_{MBP} cells were counted in longitudinal sections of optic nerve prepared, 3, 7, 14 and 21 days after injury. No significant different was observed in numbers of T_{MBP} and T_{OVA} cells in injured optic nerve (Fig. 4), indicating that antigen specificity has little to do with the accumulation of T-cells in CNS injury sites. T_{MBP} cells were detectable slightly earlier than T_{OVA} cells in the optic nerve injury site, and antigen specificity may play a role in this but is not sufficient to explain the large accumulation of T_{OVA} cells in the site of injury.

Fig. 5 shows accumulation of T-cells measured immunocytochemically using antibodies to T-cell receptors. This detection technique rules out the possibility that the observed labeling is due to phagocytic cells which had phagocytized the pre-labeled T-cells shown in Fig. 1. The graph shows a striking elevation in T-cell accumulation following injury, regardless of whether the systematically injected T-cells are specific to a self-epitope (MBP) or to a non-self epitope (OVA).

Fig. 6 shows that accumulation of T-cells is dependent on the lesion and not the breakdown of the blood-brain barrier. T-cells specific to either MBP or OVA were injected 2 weeks after injury and their accumulation analyzed a week later, namely 21 days following the primary lesion. Their accumulation was compared to that of T-cells injected immediately after injury and detected either 7 or 21 days later. It appears that the time elapsed between the injury and the injection of T-cells, which is a factor in the sealing of the blood-brain barrier, is not a factor in the T-cell accumulation.

CLAIMS:

1. A method for the delivery of a substance to a site of damage in an individual's central nervous system (CNS), comprising activating T–cells which either carry or can produce said substance, and allowing said cells to migrate within the individual's body to reach said site.

2. A method according to Claim 1, comprising treating T–cells *in vitro* so as to cause them to either carry or produce said substance, and then activating said T–cells.

3. A method according to Claim 2, comprising loading T–cells with said substance.

4. A method according to Claim 2, comprising treatment of the T–cells by inserting into the cells a DNA sequence which either encodes said substance, encodes an enzyme which catalyzes said substance, or encodes a regulatory product the production of which brings to expression of said substance in the T–cells, the DNA sequence being under expression control such that they are either expressed continuously or induced to express as a result of exposure of the T–cells to a microenvironment of a kind present at the site of DNA damage.

5. A method for diagnosis of damage in the CNS of an individual, comprising inoculating the individual with activated T–cells carrying a detectable marker substance, allowing time for migration of the T–cells to a site of damage in the CNS and then detecting the presence and location of the marker substance in the individual's CNS.

6. A method for the treatment of damage in an individual's CNS comprising inoculating the individual with activated T–cells carrying or producing a substance in need at a site of CNS damage to ameliorate the damage or symptoms associated therewith.

7. T–cells carrying or capable of producing a substance in need at a site of CNS damage, said substance being a substance which is not naturally carried or produced by the T–cells.

8. Cells according to Claim 7, which have been loaded *in vitro* by said substance.

9. Cells according to Claim 7, the cells carrying an introduced DNA sequence which either encodes said substance, encodes an enzyme which catalyzes said substance, or encodes a regulatory product the production of which brings to expression of said substance in the T-cells, the DNA sequence being under expression control such that it is either expressed continuously or induced to expression as a result of exposure of the T-cells to a micro environment of a kind present at the site of DNA damage.

For the Applicants,
DR. REINHOLD COHN AND PARTNERS
By:

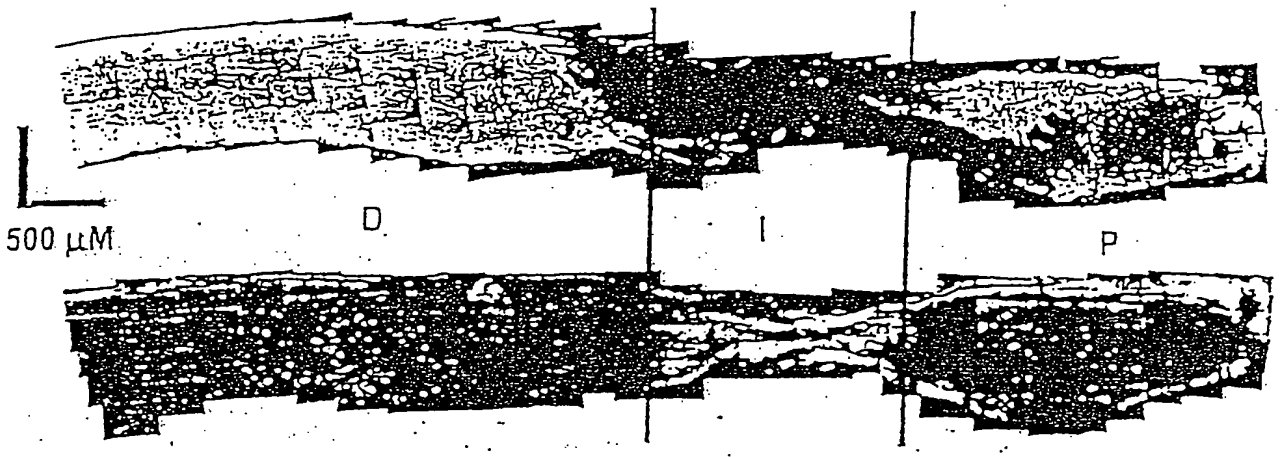


FIGURE 1

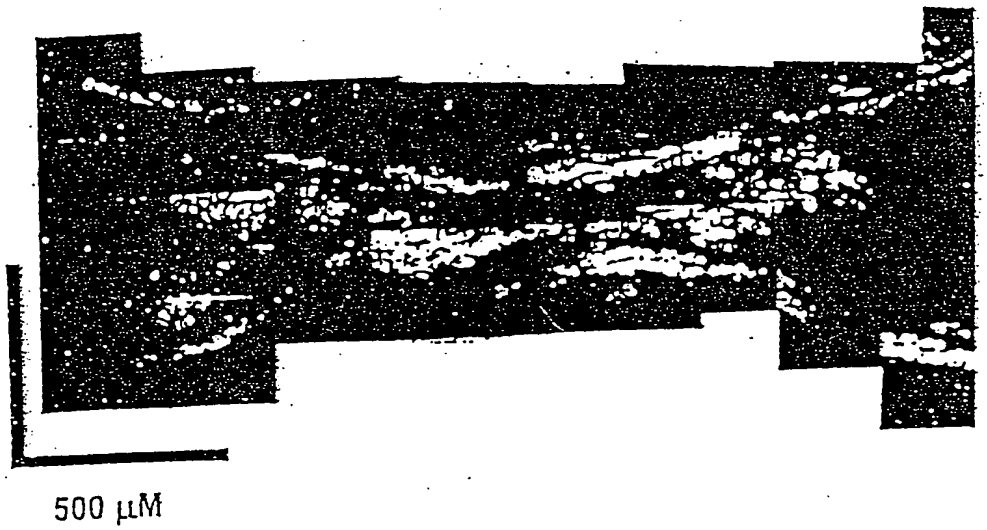


FIGURE 2

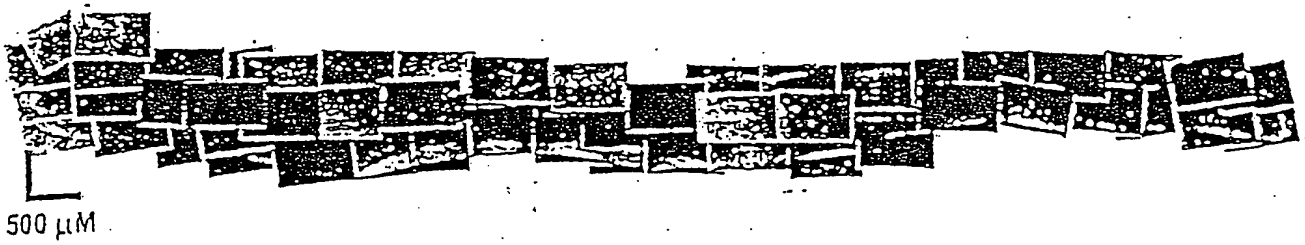


FIGURE 3

Figure 4

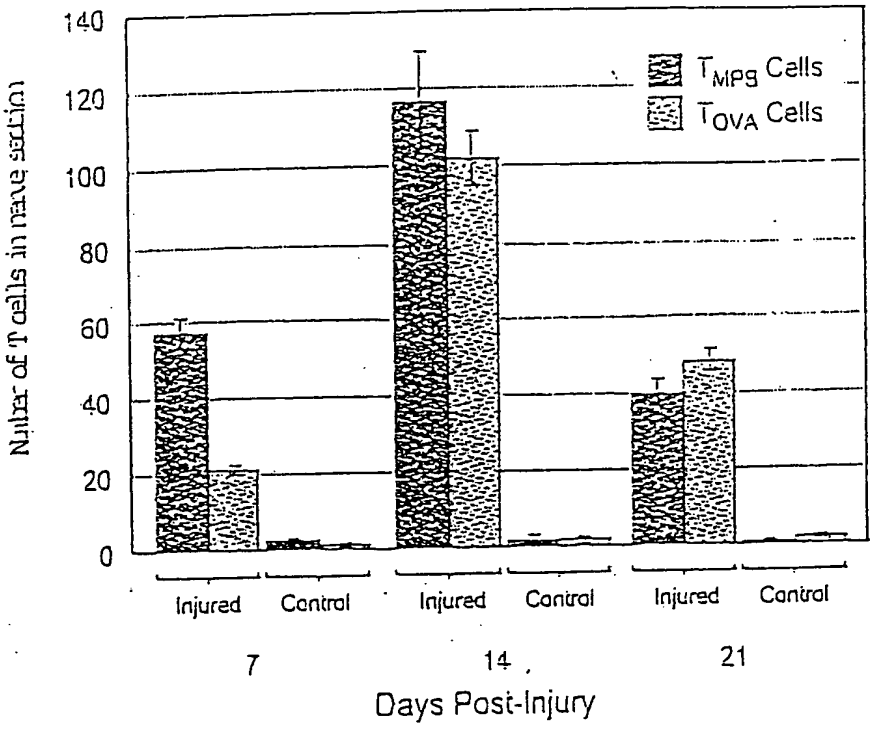
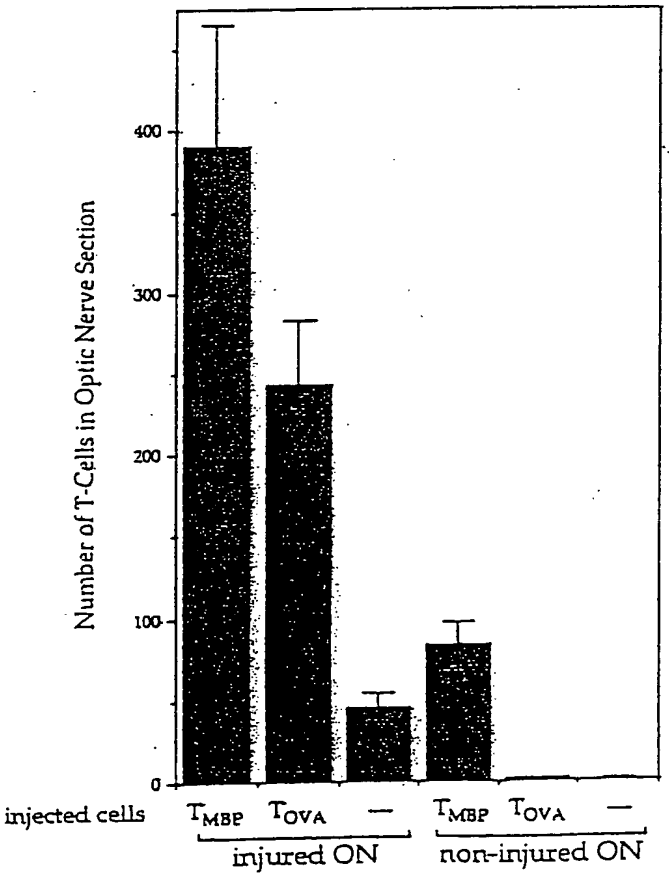


Figure 5



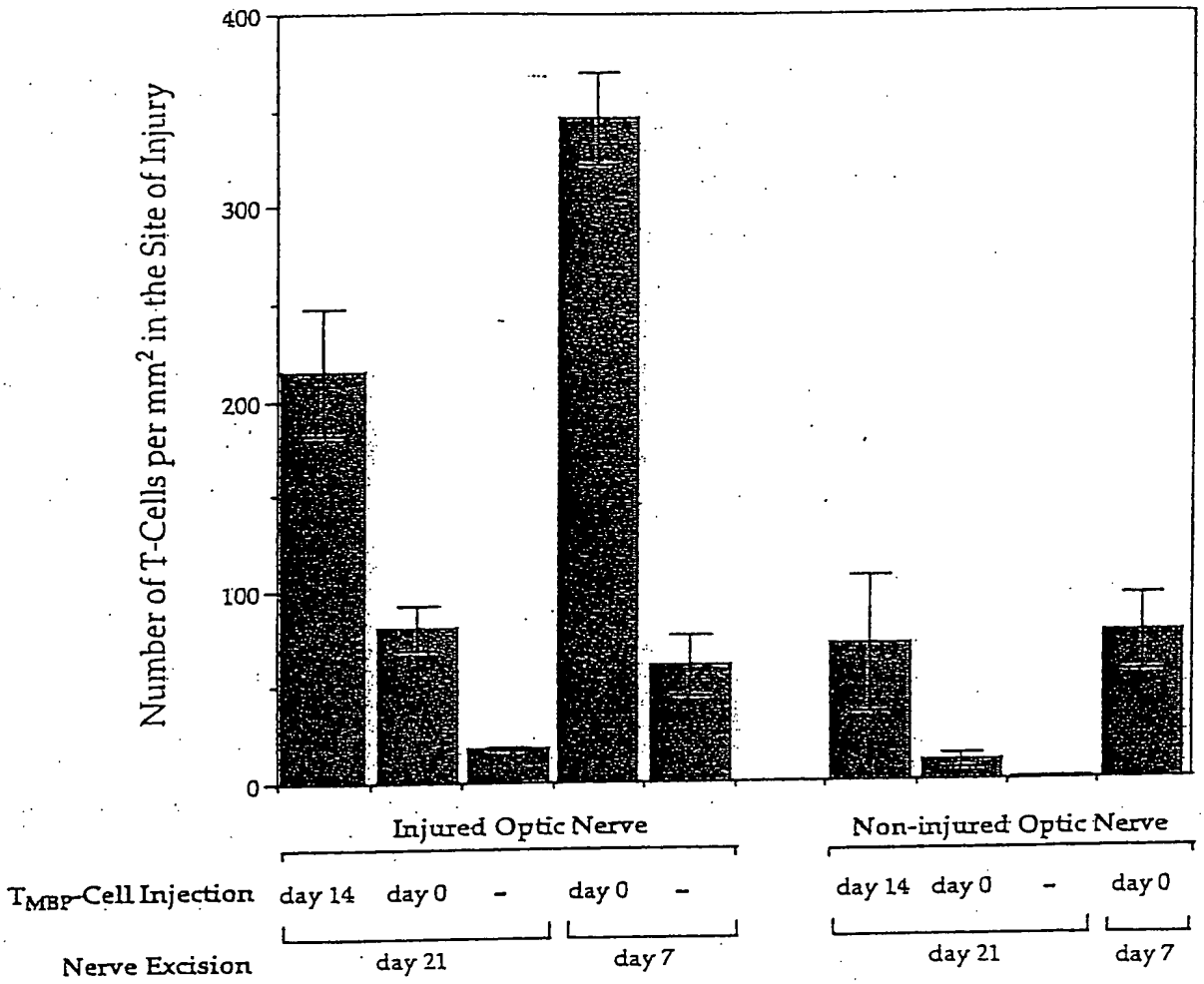

Number of T-Cells per mm² in the Site of Injury
400
300
200
100
0
Injured Optic Nerve
Non-injured Optic Nerve
T_MBP-Cell Injection
day 14
day 0
-
day 0
-
day 14
day 0
-
day 0
Nerve Excision
day 21
day 7
day 21
day 7

FIGURE 6